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No involvement of nicotinic receptors in the facilitation of acetylcholine outflow in mouse cortex in the presence of neostigmine and atropine

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1 The role of nicotinic and muscarinic receptors in the modulation of acetylcholine release was studied using field stimulated mouse cortex slices incubated with [^{3}H]-choline.

2 Both acetylcholine (100 μM) and the cholinesterase inhibitor neostigmine (100 μM) inhibited the stimulation-induced (S-I) outflow of radioactivity but in the presence of atropine (0.3 μM) an enhancement was seen, which may be indicative of facilitatory nicotinic receptors.

3 Mecamylamine (100 μM) was unable to antagonize the enhancement seen in the presence of acetylcholine and atropine. The nicotinic agonist dimethylphenylpiperazinium (30 μM) did not facilitate S-I outflow of radioactivity.

4 A range of nicotinic blockers had no effect on the enhancement seen in the presence of neostigmine and atropine, nor did indomethacin, the $5HT_3$ antagonist MDL 7222 nor the NMDA antagonist MK-801.

5 The inability to block this effect suggests that nicotinic receptors are not involved. We postulate, at least for neostigmine, that the facilitation is an artefact because of the use of [^{3}H]-choline as a radiotracer whereby the efflux of radioactivity is enhanced because the radiolabelled acetylcholine is not metabolized to choline and therefore flows out of the tissue more readily.
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Keywords: Acetylcholine release; presynaptic receptors; nicotinic receptors; muscarinic receptors; neostigmine

Abbreviations: ACH, acetylcholine; ATR, atropine sulphate; BET, bethanechol chloride; BUN, α-bungarotoxin; COB, α-cobratoxin; DMPP, 1,1-dimethyl-4-phenylpiperazinium iodide; IND, indomethacin; MEC, mecamylamine HCl; MDL, MDL 72222 (3-tropanyl-3,5-dichlorobenzoate); MK, MK 801; MLA, methyllycaconitine citrate; NEO, neostigmine methylsulphate; NMC, N-methylcarbamylcholine; PSS, physiological salt solution; S-I, stimulation-induced; TUB, tubocurarine

Introduction

Nicotinic acetylcholine receptors belong to the superfamily of ligand gated receptors and are activated by acetylcholine. They are pentamers with α, β, γ, δ, ε subunits being described to date (see Lindstrom *et al*., 1995). Multiple isoforms have been described for most subunits. Neuronal nicotinic receptors, on the other hand, are quite distinct from those located in the periphery, in that they are composed of only the α and β subunits (Changeux *et al*., 1998). The intrinsic pore of the nicotinic receptor is permeable to Na^+, Ca^{2+} and K^+ to different extents depending on subunit composition, and the different subunits may combine to form many pharmacologically distinct receptors (see Lindstrom *et al*., 1995). Their activation depolarizes cell bodies (Mike, 1994) and nerve terminals (Clarke, 1993) which results in transmitter release from a wide variety of neural types. As far as acetylcholine release is concerned, nicotinic agonists induce the release of acetylcholine from guinea-pig myenteric plexus (Briggs & Cooper, 1982; Mike, 1994), guinea-pig ileum (Soejima *et al*., 1993; Dietrich & Kilbinger, 1995), guinea-pig cortex (Beani *et al*., 1985), rat hippocampus and cortex (Araujo *et al*., 1988), rat cerebellum (Lapchak *et al*., 1989) and rat cortical minces (Meyer *et al*., 1987). Although less well established, nicotinic agonists facilitate the action-potential evoked release of acetylcholine from rat hippocampus (Araujo *et al*., 1988), guinea-pig cortex (Beani *et al*., 1985), rat cortex (Loiacono & Mitchelson, 1990; Marchi & Raiteri, 1996), mouse brain synaptosomes (Rowell & Winkler, 1984) and rat phrenic nerve (Wessler *et al*., 1986).

The most prominent presynaptic effect of acetylcholine on cholinergic neurones is an inhibitory effect through activation of presynaptic muscarinic autoreceptors, but there are also indications of facilitatory nicotinic autoreceptors (see Starke *et al*., 1989; Vizi & Lendvai, 1999). Thus, when presynaptic muscarinic receptors are blocked, cholinesterase inhibitors elevate stimulation-induced (S-I) acetylcholine release in rat cortex (Richardson & Szerb, 1974; Bourdois *et al*., 1974; Marchi & Raiteri, 1996) and guinea-pig myenteric plexus (Mike, 1994). It is possible that this effect is because the levels of acetylcholine are high enough to activate release modulating or release inducing nicotinic receptors (see above). However, some studies to date have not been able to antagonize this action with nicotinic blockers such as mecamylamine (Mike, 1994). Given the many possible subtypes of nicotinic receptors, it may be that the susceptibility to blockade of nicotinic autoreceptors is different from classical nicotinic receptors. For example, the facilitatory effect of nicotine on S-I acetylcholine release in rat cortex (Loiacono & Mitchelson, 1990) and guinea-pig

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cortex (Beani *et al*., 1985) is blocked by tubocurarine, a muscle type nicotinic blocker, and the facilitatory effect of the nicotinic agonist dimethylphenylpiperazinium iodide (DMPP) on S-I acetylcholine release in rat phrenic nerve is resistant to blockade by hexamethonium (Wessler *et al*., 1986). The aim of the present study was to investigate further the possibility of nicotinic autoreceptors using a wide variety of nicotinic blocking drugs capable of blocking a diverse range of nicotinic receptors. The study was carried out in mouse cortex slices incubated with [^{3}H]-choline.

Methods

Radioactive outflow from mouse cortex slices incubated with [^{3}H]-choline

Outbred male Swiss white mice (20–30 g) were decapitated and their brains rapidly removed and immersed in ice cold physiological salt solution (PSS). Horizontal cerebral cortex slices (400 μm thick) were prepared using a Campden Vibroslice 752. The cortical section (frontal) was isolated from either the left or right hemisphere of the slice and incubated with [^{3}H]-choline (85.1 Ci mmol^{-1}, 0.2 μM) for 30 min in PSS, oxygenated with a mixture of 5% CO_2, 95% O_2 and maintained at 37°C. Following the incubation period, the cortical slices were transferred to flow cells (four cells connected in series), one slice per cell, and continuously superfused at the rate of 1 ml min^{-1} with PSS containing hemicholinium-3 (10 μM) to prevent the reuptake of [^{3}H]-choline back into the nerve terminals (Umeda & Sumi, 1990). The slices were superfused for 92.5 min before sample collection began (washing period). Sixty minutes after the commencement of the washing period, an electrical priming stimulation was delivered through a pair of parallel platinum electrodes placed on either side of the brain slice (5 V cm^{-1} square wave pulses at a frequency of 3 Hz, 20 mA, 2 ms pulse duration, 2 min). After the washing period was completed, superfusate fractions were collected in 2.5 min samples. The total duration of the collection phase was 65 min. In each experiment the slices were field stimulated (5 V cm^{-1} square wave pulses, 3 Hz, 20 mA, 2 ms duration, 2 min) at 7.5 and 47.5 min after the start of sample collection. The effect of drugs on electrical stimulation-induced (S-I) outflow of radioactivity was determined by adding them to the superfusate 20 min before the second stimulation. When agonist and antagonist interaction was being investigated, the antagonist was added 25 min and the agonist 20 min prior to the second stimulation. At the completion of the experiments the radioactive content of the superfusate solution was determined by the addition of 3 ml Picofluor 40 (Packard Instruments, Melbourne, Australia) and by liquid scintillation counting. The results were expressed as disintegrations per minute (d.p.m.) after automatic external standardization. When DMPP was used, it was added to the superfusate to reach the brain slice 30 s before the second stimulation and the collection time was reduced to every 30 s instead of 2.5 min.

Separation of [^{3}H]-choline and [^{3}H]-acetylcholine using high performance liquid chromatography

Cerebral cortex slices were prepared as described previously. They were incubated with [^{3}H]-choline and then followed the same protocol as described for release experiments until the end of the washing period was completed (total of 90 min). At this point the flow rate was decreased to 0.5 ml min^{-1} and the superfusate fractions were collected in 1 min samples. The total duration of the collection phase was 30 min. In each experiment the slices were field stimulated 25 min after the start of collection. Drugs were added to the superfusate from 25 min (atropine) or 20 min (neostigmine, acetylcholine), prior to the stimulation. The amount of choline and acetylcholine in each sample was determined by high performance liquid chromatography (HPLC). A reverse-phase column was used (Excsil ODS-3). The elution solvent consisted of 0.05 M KH_2PO_4 adjusted to pH 7 with K_2HPO_4 with 250 mg l^{-1} of sodium octyl sulphate, 1.1 ml l^{-1} of tetramethylammonium chloride and methanol 10% v v^{-1}. The reaction times of choline (7–9 min) and acetylcholine (12–14 min) were determined using radioactive standards ([^{3}H]-choline and [^{14}C]-acetylcholine).

[^{3}H]-choline and [^{14}C]-acetylcholine accumulation in mouse cortex slices

Cerebral cortex slices were prepared as described above except that uniform sized slices were made using a tissue punch. The cortical sections were then placed in 1 ml PSS containing neostigmine (100 μM), a cholinesterase inhibitor, and hemicholinium-3 (10 μM). This was oxygenated with a mixture of 5% CO_2, 95% O_2 and maintained at 37°C for 15 min. Following the pre-incubation period, either [^{3}H]-choline (1–30 μM) or [^{14}C]-acetylcholine (3–30 μM) was added for 2 min. The slices were then removed and rinsed in 50 ml of PSS and each dissolved in 0.5 ml of Soluene (Packard Instruments, Melbourne, Australia) before liquid scintillation counting.

Calculation of results for release studies

For each stimulation, the basal (spontaneous) outflow was taken as the mean of the radioactive content of the sample taken during the 2.5 min period immediately before and the 2.5 min period commencing 10 min after the start of field stimulation. The S-I outflow of radioactivity was calculated by subtracting the mean basal outflow from the radioactive content of each of the four samples collected immediately after the commencement of the stimulation. With DMPP experiments there were minor variations due to different sample collection rates (30 s samples) but the procedure was similar. In all experiments, both the basal and S-I outflow in the second stimulation period were expressed as a percentage of that in the first stimulation period (B_2 as a percentage of B_1 and S_2 as a percentage of S_1 respectively).

Statistical analysis

All data are expressed as means and standard errors of the means (mean $\pm$ s.e.mean), the number of slices used is indicated by *n*. In instances of multiple comparison against a single control, the data were analysed using one-way ANOVA followed by a Dunnett's test or a two-way ANOVA. Where a single comparison was made against a single control, the data were analysed by Student's *t*-test. In instances where Student's *t*-test was inappropriate because of non-homogeneous variance, Mann–Whitney *U*-Test was used. The statistical package GB-STAT (Dynamic Microsystems, Silver Spring, U.S.A.) was used for statistical analyses. Probability levels of less than 5% ($P<0.05$) were taken to indicate statistical significance.

Materials

The physiological salt solution contained (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.03, $NaHCO_3$ 25, D-glucose 11.1, $MgSO_4$ 1.2, $CaCl_2$ 1.3, and disodium EDTA 0.067.

Radiochemicals and drugs

Drugs used were [methyl-^{3}H]-choline chloride (specific activity 85.1 Ci mmol^{-1}) and [^{14}C]-acetylcholine iodide (specific activity 4.2 mCi mmol^{-1}) (Dupont NEN Products, Boston, U.S.A.). Acetylcholine perchlorate, atropine sulphate, bethanechol chloride, α-bungarotoxin, 1,1-dimethyl-4-phenylpiperazinium iodide, hemicholinium-3, mecamylamine HCl, neostigmine methylsulphate and tubocurarine were obtained from Sigma, St Louis, U.S.A. α-Cobratoxin, MDL 72222 (3-tropanyl-3,5-dichlorobenzoate) methyllycaconitine citrate, MK 801 ((+)-5-methyl-10,11-dihydro-5H-dibenzo [a,d] cyclohepten-5,10-imine maleate) and N-methylcarbamylcholine chloride were obtained from Research Biochemicals Inc, U.S.A. Indomethacin was obtained from Merck–Sharp–Dohme (Sydney, Australia). Stock solutions of all drugs, except for acetylcholine, neostigmine and atropine, were made and kept at −20°C. On the day of the experiment, the drugs were thawed and further diluted to the appropriate concentration in physiological salt solution. Indomethacin was dissolved first in ethanol and then diluted down to the appropriate concentration in PSS. All drugs were dissolved directly in PSS.

Results

Radioactive outflow from mouse cortex incubated with [^{3}H]-choline

[^{3}H]-choline was incorporated into the cholinergic stores of mouse cortex slices and the electrical field stimulation evoked a stimulation-induced (S-I) outflow of radioactivity which was taken as an index of acetylcholine release. There were two periods of electrical stimulation (S_1 and S_2, both at 3 Hz for 2 min). The S-I outflow in the first period (S_1) was 4617±1435 d.p.m. ($n=7$), and the basal outflow (B_1) was 1304±143 d.p.m. ($n=7$) in the first series. Both tetrodotoxin (0.3 μM) and calcium omission (after addition of the Ca^{2+} chelator EGTA, 100 μM) from the superfusion medium during the second stimulation period almost abolished S-I transmitter outflow in the second stimulation (S_2 as a percentage of S_1: control = 47.3±2.7%, $n=7$; tetrodotoxin = 9.3±6.1%, $n=4$; zero Ca^{2+} = 2.8±2.5%, $n=8$, ($P<0.05$ in either case, Dunnett's test). These results indicate that transmitter was being released by propagated action-potentials and involved Ca^{2+} entry at the nerve terminal. Neither tetrodotoxin nor Ca^{2+} omission affected basal radioactive outflow (data not shown).

Effect of cholinergic drugs on radioactive outflow

Acetylcholine, bethanechol and the nicotinic receptor selective N-methylcarbamylcholine concentration-dependently inhibited the S-I outflow of radioactivity from mouse cortical slices incubated with [^{3}H]-choline with the largest effect being observed at 100 μM (Figure 1). There was also an increase in the basal outflow of radioactivity with acetylcholine, but not N-methylcarbamylcholine bethanechol (Figure 1). Atropine (0.3 μM) alone did not have any significant effect on S-I transmitter outflow (Figure 2) indicating no automodulation by endogenously released acetylcholine. However, when acetylcholine (100 μM) and atropine were added in combination, S-I transmitter outflow was significantly elevated above both drug-free control and atropine alone (Figure 2). The nicotinic antagonist mecamylamine (100 μM) was unable to antagonize the facilitation of S-I outflow by the combination of acetylcholine and atropine (Figure 2). Alone, mecamylamine did not have any effect on S-I radioactive outflow, nor did it have any effect on basal radioactive outflow or the enhanced basal outflow induced by acetylcholine (Figure 2).

Effect of neostigmine on radioactive outflow

Neostigmine, a cholinesterase inhibitor, concentration-dependently inhibited the S-I outflow (Figure 3) of radioactivity

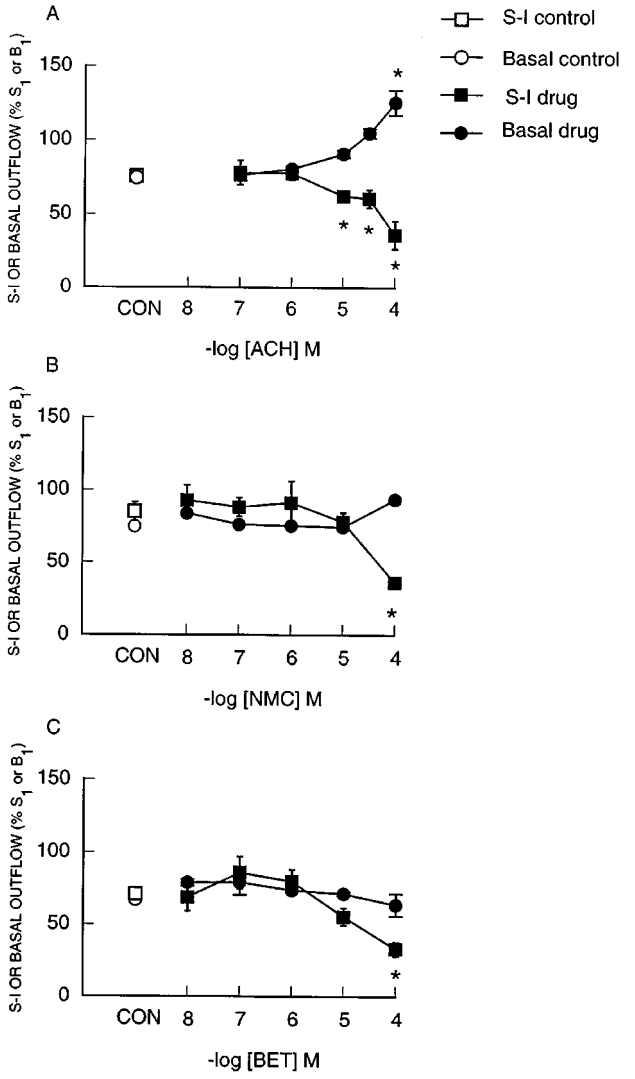

Figure 1 The effect of acetylcholine (ACH; Panel A) and N-methylcarbamylcholine chloride (NMC; Panel B) and bethanechol (BET; Panel C) on the stimulation-induced (S-I) and basal outflow of radioactivity from mouse cortex incubated with [^{3}H]-choline. There were two stimulation periods (each at 3 Hz for 2 min) 40 min apart, and the drugs were present only for the second stimulation period. The mean S-I outflow in the second stimulation period (S_2), expressed as a percentage of the first stimulation period (S_1), and the mean basal outflow in the second stimulation period (B_2), expressed as a percentage of that in the first stimulation period (B_1), are shown. The symbols represent the mean and the vertical lines represent the standard error of the mean (s.e.mean). The number of slices used for each concentration are between 6 and 64. *Represents a significant difference from control (CON; $P<0.05$, Dunnett's test, after one-way ANOVA).

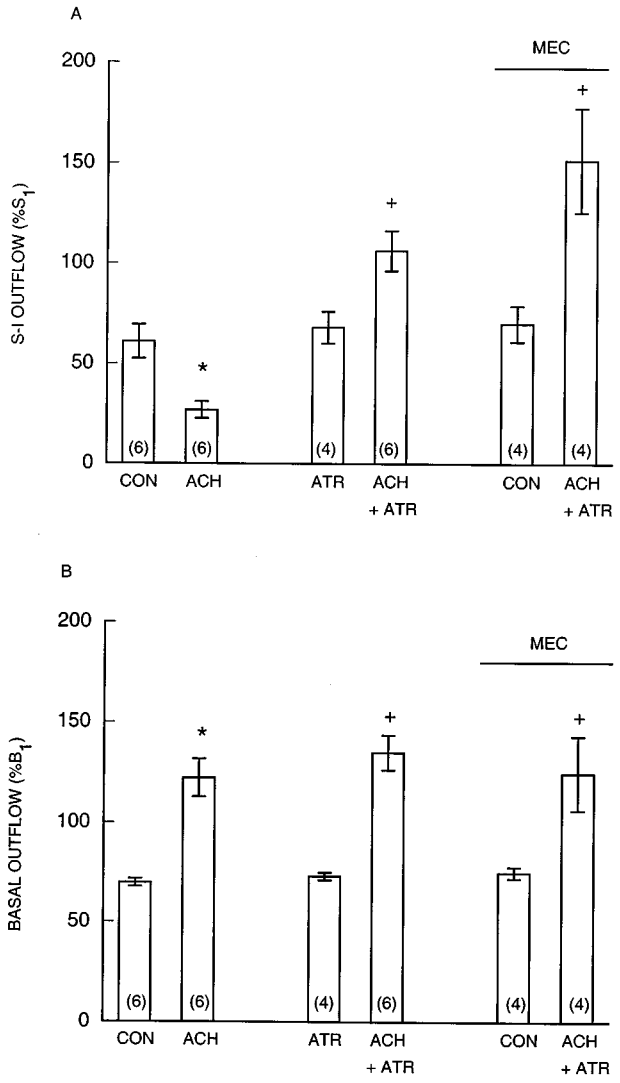

Figure 2 The effect of mecamylamine (MEC; 100 μM) on the facilitatory effect of the combination of atropine (ATR; 0.3 μM) and acetylcholine (ACH; 100 μM), on the S-I outflow of radioactivity from mouse cortex incubated with [^{3}H]-choline. There were two stimulation periods (each at 3 Hz for 2 min) 40 min apart, and drugs were present only for the second stimulation period. All results are expressed as described in Figure 1 legend. The columns represent the mean and the vertical lines represent the s.e.mean, and the number of slices used for each concentration are shown in parentheses in each column. *Represents a significant difference from experiments done in the absence of any drugs (CON; $P<0.05$. Dunnett's test, after one-way ANOVA). +Represents a significant difference from experiments done in the presence of atropine (ATR) or mecamylamine (CON) alone ($P<0.05$, Student's t-test).

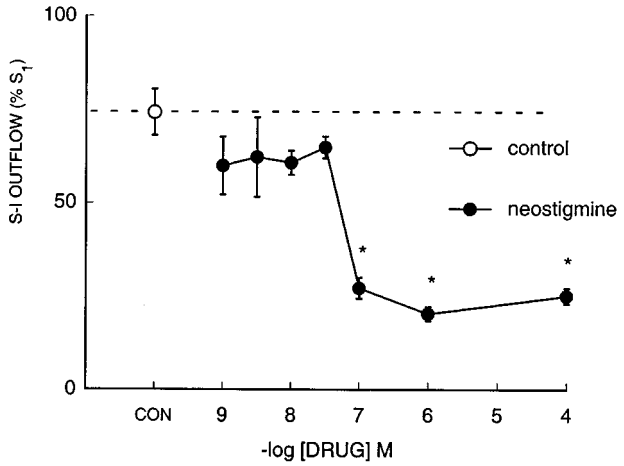

Figure 3 The effect of neostigmine (NEO) on the S-I outflow of radioactivity from mouse cortex incubated with [^{3}H]-choline. There were two stimulation periods (each at 3 Hz for 2 min) 40 min apart, and neostigmine was present only for the second stimulation period. All results are expressed as described in Figure 1 legend. The number of slices used for each concentration are between 4 and 18. *Represents a significant difference from control (CON; $P<0.05$, Dunnett's test, after one-way ANOVA).

Table 1 The effect of nicotinic antagonists on the S-I outflow of radioactivity from mouse cortex in the presence of atropine and neostigmine

	S_2 (%S_1)
CON	70.3±2.9
NEO (100 μM)	25.1±1.4*
ATR (0.3 μM)	78.9±4.3
ATR+NEO	128.4±8.4*
MEC (30 μM)	61.9±7.7
TUB (1 μM)	65.9±6.3
BUN (1 μM)	76.9±7.8
COB (0.3 μM)	71.9±2.3
MLA (3 μM)	73.9±5.2
ATR+NEO+MEC (30 μM)	122.0±15.3†
ATR+NEO+TUB (1 μM)	112.1±6.0†
ATR+NEO+BUN (1 μM)	141.7±13.6†
ATR+NEO+COB (0.3 μM)	126.6±21.3†
ATR+NEO+MLA (3 μM)	144.6±12.7†

There were two stimulation periods (each at 3 Hz for 2 min) 40 min apart, and drugs were present only for the second stimulation period. All results are expressed as described in Figure 1 legend. In some experiments mecamylamine (MEC); tubocurarine (TUB); α-bungarotoxin (BUN); α-cobratoxin (COB) and methyllyaconitine (MLA) were also present in S_2 in order to determine their effects on the facilitation of acetylcholine outflow in the presence of atropine (ATR) and neostigmine (NEO). *Represents a significant difference from control (CON; $P<0.05$, Dunnett's test, after one-way ANOVA). †Represents a significant difference from the respective nicotinic antagonist alone ($P<0.05$, Dunnett's test, after one-way ANOVA).

without any significant effect on basal radioactive outflow (not shown). The inhibition of S-I radioactive outflow in the presence of neostigmine (100 μM) was blocked by atropine (0.3 μM) and the S-I outflow for the combination of atropine and neostigmine was greater than drug-free control (Table 1), however, basal outflow was not affected. This result suggests that neuronally-released acetylcholine can activate inhibitory muscarinic receptors and when these are blocked with atropine, a facilitatory effect is revealed.

Effect of DMPP on radioactive outflow

The nicotinic agonist DMPP (30 μM), did not significantly affect either the basal or S-I outflow of radioactivity (78.2±2.7 and 65.9±5.1%, respectively) from cortical slices following a brief exposure time (30 s).

Facilitation of radioactive outflow: effect of nicotinic antagonists

A range of nicotinic antagonists were used to determine which receptor was involved in the facilitatory effect on S-I outflow in the presence of neostigmine and atropine (Table 1). The antagonists used were: mecamylamine (30 μM); tubocurarine

(1 μM); α-bungarotoxin (1 μM); α-cobratoxin (0.3 μM) methyllycaconitine (3 μM). None of these antagonists affected the S-I outflow and none affected the facilitatory effect of the combination of neostigmine and atropine (Table 1). No effects were seen on basal radioactive outflow (not shown).

Effect of MDL 72222 and MK801 on radioactive outflow

Two other receptor antagonists were used to investigate whether another receptor type was involved in the facilitation of S-I outflow seen with the combination of atropine and neostigmine. The antagonists used were: MDL 72222, a 5-HT_3 receptor antagonist (1 μM), and MK 801 (1 μM), a non-competitive ligand gated channel blocking agent. Neither antagonists affected the S-I outflow (Table 2) and neither affected the facilitatory effect of the combination of neostigmine and atropine (Table 2). There was no significant effect on basal radioactive outlfow (not shown).

Effect of indomethacin on radioactive outflow

These experiments were all done in the presence of ethanol (0.035%) as this was the vehicle for indomethacin. Indomethacin (10 μM) alone had no significant effect on S-I outflow of transmitter (Table 2), nor did it have any significant effect on the facilitatory effect of the combination of neostigmine and atropine.

Tissue accumulation of choline and acetylcholine

Cortical slices were treated with neostigmine (100 μM) and the choline uptake inhibitor hemicholinium-3 (10 μM) and the uptake of [^{3}H]-choline and [^{14}C]-acetylcholine measured over 2 min to mimic the time frame of the release of transmitter. The uptake of choline was about 200 fold greater than the uptake of acetylcholine (Figure 4).

Table 2 The effect of noncholinergic antagonists on the S-I outflow of radioactivity from mouse cortex in the presence of atropine and neostigmine

	S_2 (%S_1)
CON	70.3±2.9
NEO (100 μM)	25.1±1.4*
ATR (0.3 μM)	78.9±4.3
ATR+NEO	128.4±8.4*
MDL (1 μM)	56.1±8.5
MK (1 μM)	68.9±17.6
ATR+NEO+MDL (1 μM)	112.6±10.2†
ATR+NEO+MK (1 μM)	131.9±11.2†
In the presence of ethanol	
CON	64.5±7.2
ATR+NEO	101.5±6.9*
IND (10 μM)	57.4±3.5
ATR+NEO+IND (10 μM)	87.9±9.1†

There were two stimulation periods (each at 3 Hz for 2 min) 40 min apart, and drugs were present only for the second stimulation period. In some experiments MDL 72222 (MDL), MK 801 (MK) and indomethacin (IND) in order to determine their effects on the facilitation of acetylcholine outflow in the presence of atropine (ATR) and neostigmine (NEO). All results are expressed as described in Figure 1 legend. *Represents a significant difference from respective control (CON; $P<0.05$, Dunnett's test, after one-way ANOVA). †Represents a significant difference from the respective antagonist experiments ($P<0.05$, Dunnett's test, after one-way ANOVA).

Effect of neostigmine or acetylcholine in combination with atropine on S-I [^{3}H]-choline and [^{3}H]-acetylcholine outflow

In the presence of atropine (0.3 μM) alone, the basal and S-I outflow consisted almost entirely of [^{3}H]-choline (Figure 5).

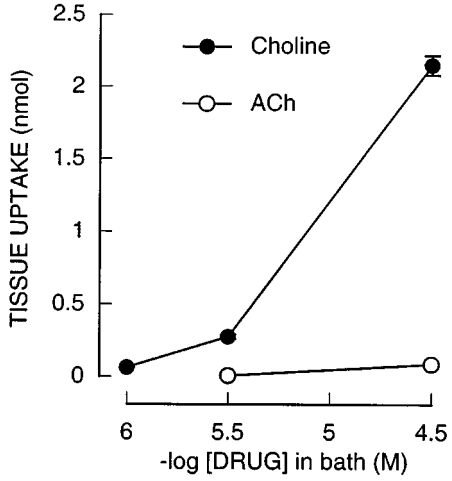

Figure 4 The uptake of [^{3}H]-choline and [^{14}C]-acetylcholine in slices of rat cortex during 2 min incubation with radioligand. These experiments were performed in the presence of neostigmine (100 μM) and hemicholinium-3 (10 μM). The mean and s.e.mean are shown. The number of slices used for each concentration are between 4 and 8.

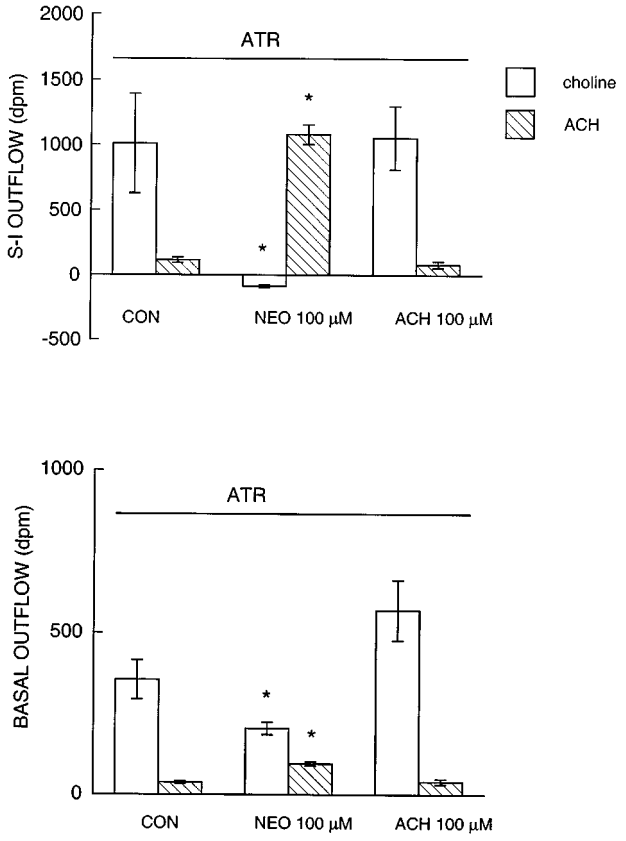

Figure 5 The effect of the combination of atropine (ATR; 0.3 μM) and neostigmine (NEO; 100 μM) or acetylcholine (ACH; 100 μM) on the S-I outflow of [^{3}H]-choline and [^{3}H]-acetylcholine as assessed by HPLC. The columns represent the mean and the vertical lines represent the s.e.mean., and the number of slices used for each group are between 3 and 7. *Represent a significant difference from the respective control (CON; $P<0.05$, Mann–Whitney U-Test).

This was not altered by the addition of exogenous acetylcholine (Figure 5). However, neostigmine (100 μM) increased the proportion of [^{3}H]-acetylcholine in both the basal and S-I outflows (Figure 5).

Discussion

In the present study, both acetylcholine (100 μM) and bethanechol (100 μM) inhibited the S-I radioactive outflow from mouse cortical slices incubated with [^{3}H]-choline and in the presence of atropine (which by itself had no effect on S-I radioactive outflow), the inhibition seen with acetylcholine was reversed to a facilitatory effect. A similar finding has been observed in rat hippocampus where the inhibitory effect of carbachol on acetylcholine release was reversed to a facilitatory effect in the presence of atropine (Hadházy & Szerb, 1977). One explanation for this is that in addition to inhibitory muscarinic receptors, cholinergic terminals possess facilitatory nicotinic receptors which can be activated by acetylcholine. Indeed, cholinergic terminals do possess nicotinic receptors which are release inducing and release enhancing (see Introduction). However, it should be noted that N-methyl, carbamylcholine, a relatively selective nicotinic agonist, did not enhance the S-I outflow of radioactivity, the only effect being an inhibition at the highest concentrations tested. Furthermore, the nicotinic agonist DMPP did not induce release on its own, nor did it facilitate the S-I outflow of radioactivity, suggesting that there was no involvement of nicotinic receptors. We carried out this experiment with a short contact time (30 s) since it has been demonstrated by others that neuronal nicotinic receptors rapidly desensitize (Beani *et al*., 1985). Furthermore, this contact time has been demonstrated to be sufficient to enhance transmitter outflow (Wessler *et al*., 1987). This further supports the absence of presynaptic nicotinic receptors in this study and also excludes possible nicotinic desensitization induced by the other nicotinic agonists used in this study.

It is possible that neurally released acetylcholine can activate a facilitatory process since, in the present study, when cholinesterase was inhibited with neostigmine and presynaptic muscarinic receptors blocked by atropine, the S-I outflow of acetylcholine was significantly greater than that of atropine alone. This has been observed in other preparations including rat cortex (Richardson & Szerb, 1974; Bourdois *et al*., 1974; Marchi & Raiteri, 1996), and guinea-pig myenteric plexus (Mike, 1994). If a positive feedback system exists, there are several possible receptors which may be involved. Firstly, it is unlikely that the effects observed are due to facilitatory muscarinic receptors since atropine is a potent antagonist at all muscarinic receptor subtypes (Mei *et al*., 1989). A more likely explanation is that this effect is occurring through release modulating or release inducing nicotinic receptors (see above). However, a number of studies using similar stimulation parameters such as used in the present study, have not been able to antagonize this action with nicotinic blockers such as mecamylamine (Mike, 1994).

In the present investigation, there was no blocking effect of the facilitation seen after neostigmine and atropine with a variety of nicotinic antagonists used at concentrations capable of, or exceeding those, which block neuronal nicotinic effects in other tissues: mecamylamine (Alkondon & Albuquerque, 1990), α-bungarotoxin (Alkondon & Albuquerque, 1995), methyllycaconitine (Khan *et al*., 1994; Alkondon & Albuquerque, 1995), α-cobratoxin (Alkondon & Albuquerque, 1990), and tubocurarine (Araujo *et al*., 1988; Lapchak *et al*., 1989). Together these data suggest that nicotinic receptors are not involved in the facilitatory effect of neostigmine or acetylcholine seen after muscarinic blockade.

A brain slice preparation is heterogeneous and the possibility exists that substances released from other neurones or cells may participate in the facilitation of acetylcholine release. For example, the excitatory amino acid N-methyl-D-aspartate (NMDA) evoked the release of acetylcholine from rat striatal slices and this was blocked by MK-801 (Ransom & Deschenes, 1989). *In vivo* data suggest that 5-hydroxytryptamine (5-HT) may exert a regulatory control on acetylcholine release in rat dorsal hippocampus *via* 5-HT$_3$ receptors (Consolo *et al*., 1994). However, in the present study, neither MK-801, nor MDL-7222, a 5HT$_3$ antagonist, had any effect on the facilitation of S-I acetylcholine outflow due to the combination of neostigmine and atropine. Finally, the cyclooxygenase inhibitor indomethacin has been shown to inhibit DMPP-induced release of acetylcholine from guinea-pig myenteric plexus (Takeuchi *et al*., 1991) and indomethacin reduced the nicotine-induced release of acetylcholine in isolated ileal synaptosomes (Cheng & Shinozuka, 1987). However, in the present study, indomethacin was unable to antagonize the facilitation of S-I acetylcholine outflow due to the combination of neostigmine and atropine.

The lack of effect with the foregoing drugs prompted us to examine alternate hypotheses to explain the enhanced S-I outflow seen after atropine in combination with either acetylcholine or neostigmine. It may be that this facilitatory effect, which has been reported by others, is an artefact of the use of [^{3}H]-choline as a radiotracer. It is well known that when radiolabelled acetylcholine is released, it is rapidly broken down to choline and the outflow from the tissue is radiolabelled choline (Richardson & Szerb, 1974). We suggest that when breakdown of [^{3}H]-acetylcholine to [^{3}H]-choline takes place after the release of [^{3}H]-acetylcholine, a portion of the [^{3}H]-choline is sequestered in the tissue and the observed outflow into the bathing fluid underestimates the actual amount of acetylcholine released. However, when the breakdown of the released [^{3}H]-acetylcholine is prevented, the radioactive outflow consists of a larger proportion of acetylcholine which is not retained by the tissue (as we showed in the present study) and this may then appear as an apparent facilitation. Indeed, in the presence of the choline uptake inhibitor hemicholinium-3 (10 μM) which was also present in the current release experiments, the brain slice accumulation of [^{3}H]-choline from the bathing medium was several hundred fold higher than the accumulation of [^{14}C]-acetylcholine over a 2 min period. However, whilst this may explain the facilitation in the presence of neostigmine it cannot explain the facilitatory effect of acetylcholine since acetylcholine did not alter the metabolic profile and this remains a puzzle.

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